Vaccine 28 (2010) 2505–2509

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# Production of H5N1 (NIBRG-14) inactivated whole virus and split virion influenza vaccines and analysis of immunogenicity in mice using different adjuvant formulations

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ARTICLE INFO

*Article history:*
Received 16 October 2009
Received in revised form
28 December 2009
Accepted 16 January 2010
Available online 30 January 2010

*Keywords:*
H5N1 influenza vaccine
Adjuvant

ABSTRACT

Consecutive lots of H5N1 (A/Vietnam/1194/2004 – NIBRG-14) split virion and whole virus vaccines were produced in a pilot-scale laboratory. The average yields of vaccine doses (15 μg HA) per egg were 0.57 doses for H5N1 split virion vaccine and 1.12 for H5N1 whole virus vaccine, compared to 2.09 doses for the seasonal H3N2 split virion vaccine. H5N1 split virion vaccine lots complied with WHO protein content criteria, while some lots of the H5N1 whole virus vaccine showed protein content per dose higher than the limit established. All lots of both vaccines showed ovalbumin (OVA) concentration below the recommended limit. Dose sparing strategies using adjuvant formulations using aluminum hydroxide ($Al(OH)_3$) and monophosphoryl lipid A (MPLA) from *Bordetella pertussis* were tested in mice. Both 3.75 μg HA and 7.5 μg HA of H5N1 split virion vaccine with $Al(OH)_3$ or $Al(OH)_3$ plus MPLA in aqueous suspension showed higher hemagglutination-inhibition (HAI) titers when compared to the same vaccine dose without any adjuvant. Immunization with the H5N1 inactivated whole virus vaccine was also performed using 3.75 μg HA and HAI titers were higher than those induced by the split virion vaccine. Moreover, the use of $Al(OH)_3$ with MPLA as an emulsion induced a further increase in HAI titers.



## 1. Introduction

Avian H5N1 influenza infection in humans has been first reported in 1997 and a total of 442 cases have been confirmed by WHO until September 2009, with a very high case fatality rate of 60% [1]. Prepandemic H5N1 vaccines have been thus developed and tested. As part of the preparedness plan in Brazil for an influenza pandemic, H5N1 (A/Vietnam/1194/2004 – NIBRG-14) vaccines were produced in a pilot-scale laboratory using embryonated hen's eggs. Shortage of vaccine is of particular concern in developing countries, once the estimated global production capacity of seasonal influenza vaccines is of approximately 350 million doses and mostly in production plants located in industrialized countries [2]. Due to the lack of pre-existing immunity against a new pandemic virus, higher vaccine dosages will probably be required. Moreover, large amounts of doses will have to be produced within a short period. Dose sparing strategies will thus be particularly important in an influenza pandemic scenario. The yield per egg is also a concern, since H5N1 vaccines have shown lower yields than the seasonal vaccine strains.

A recent review has analyzed the results of different human trials of H5N1 vaccines and whole virus vaccines were in general more immunogenic than non-adjuvanted split virion ones. Moreover, the use of oil-in-water emulsions provided the best results when added to split virion influenza vaccines [3]. The use of adjuvants for the seasonal influenza vaccine has also been proposed by our group as a way to increase the capacity of doses produced and to reduce costs due to decrease in the antigen content of the vaccine. The adjuvant monophosphoryl lipid A (MPLA) from *Bordetella pertussis* was obtained by acid hydrolysis of LPS, which is a by-product of a new cellular pertussis vaccine with lower endotoxin content [4]. We have shown that MPLA alone or combined with aluminum hydroxide ($Al(OH)_3$) induced an increase in hemagglutination-inhibition (HAI) titers in mice immunized with H3N2 (A/Wisconsin/67/2005) split virion vaccine [5].

Here we show the production of H5N1 (A/Vietnam/1194/2004 – NIBRG-14) whole virus and split virion vaccines in eggs, with the analysis of yields in comparison to H3N2 (A/Panama/2007/99) split virion seasonal vaccine strain. Protein and ovalbumin (OVA) content in the preparations were also analyzed. Immunogenicity

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doi:10.1016/j.vaccine.2010.01.044

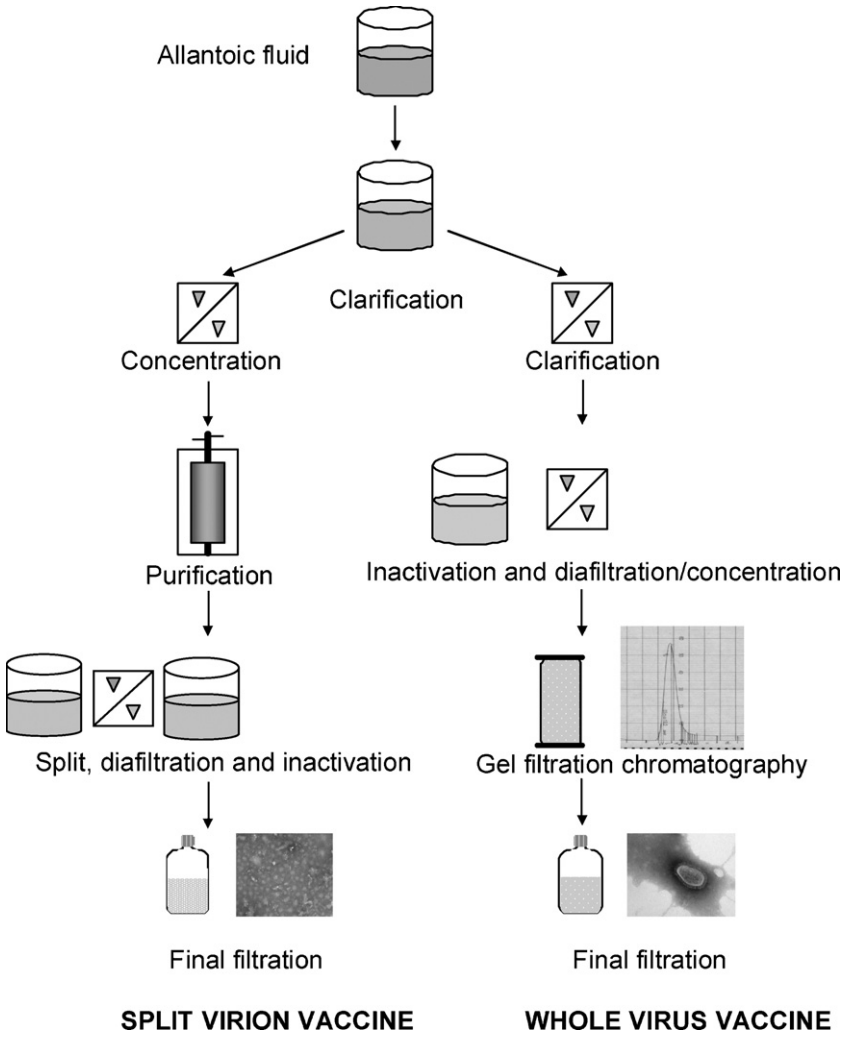


**Fig. 1.** Flow diagram for the production of split virion and whole virus influenza vaccines.

of the vaccine and the impact of the use of MPLA and $Al(OH)_3$ as adjuvants in mice are also shown.

## 2. Materials and methods

### 2.1. Vaccine production

Influenza H5N1 vaccines were produced in a pilot-scale biosafety level 3 laboratory using the A/Vietnam/1194/2004 (NIBRG-14) reference virus, provided by the National Institute for Biological Standards and Controls (NIBSC, UK). NIBRG-14 is a reassortant virus produced by reverse genetics containing the internal genes of A/PR/8/34, and hemagglutinin (HA) and neuraminidase (NA) genes from A/Vietnam/1194/04 virus and modified by replacing the polybasic amino acids at the cleavage site to render the virus avirulent [6]. H3N2 (A/Panama/2007/99) virus is a seasonal influenza vaccine strain.

For the production of the inactivated vaccines, the seed virus was grown in 11 days-embryonated hen's eggs. 72 h after the inoculation, embryos were killed by chilling the eggs. For the split virion vaccines, the harvested allantoic fluid was clarified, purified by sucrose gradient zonal centrifugations, disrupted using Triton X-100 and inactivated with formaldehyde. For the whole virus vaccines, the harvested allantoic fluid was clarified, inactivated with formaldehyde and submitted to gel filtration chromatography for concentration and purification (Fig. 1). Influenza vaccines were resuspended in phosphate buffered saline (PBS), filtered to remove bacteria and inoculated in embryonated hen's eggs in order to confirm the total inactivation of the virus.

### 2.2. Adjuvants

Alhydrogel ($Al(OH)_3$—Brenntag Biosector, Denmark) and MPLA were used as adjuvants. MPLA was produced using LPS from previously detoxified whole cell pertussis vaccine, followed by organic extraction and hydrolysis as described [5].

### 2.3. Immunization and analysis of the immune response

Female BALB/c mice weighting 18–20 g (8 weeks) were supplied by the Butantan Institute's Central Animal House. Experiments were performed according to experimental protocols approved by the Animal Use Ethics Committee of Butantan Institute. The different vaccine formulations were administered in 0.5 mL through the intraperitoneal route. Animals were bled individually and sera were used for the determination of HAI titers using horse erythrocytes, with methods routinely used for the determination of the potency of vaccines for human use. An HAI titer $\geq$1:40 is considered a correlate of protection in humans [7]. Total anti-H5N1 IgG, IgG1 and IgG2a antibody concentrations were determined in individual sera by ELISA, using 250 ng/mL HA of H5N1 split vaccine as coating antigen. Goat anti-mouse IgG, anti-mouse IgG1, anti-mouse IgG2a and